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PROVISIONAL SPECIFICATION

Invention Title: "Flavivirus Expression System"

The invention is described in the following statement:

Flavivirus Expression System

The present invention generally relates to the field of recombinant gene expression and in particular to Flavivirus expression systems.

Improved methodologies for maximising recombinant gene expression is an on-going effort in the art. Of particular interest is the development of methodologies that maximise recombinant expression of mammalian genes in safe vectors suitable for producing commercially useful quantities of biologically active proteins.

Currently, there are numerous expression systems available for the expression of genes. While prokaryotic and yeast expression systems are extremely efficient and easy to use, these systems suffer from a number of disadvantages, including an inability to glycosylate proteins, inefficient cleavage of "pre" or "prepro" sequences from proteins (eg., inefficient post translational modification), and a general inability to secrete proteins.

Another expression system widely available is the baculovirus expression system. This system is arguably one of the more efficient in protein production, but is limited only to use in insect cell lines. Unfortunately, insect cell lines glycosylate proteins differently from mammalian cell lines thus this system has not proven useful for the production of many mammalian proteins. Another disadvantage of this system is that it relies on the use of homologous recombination for the construction of recombinant virus stocks. Thus, this system often proves very laborious when large numbers of genetic variants have to be analysed.

In view of these problems the art has sought eukaryotic host systems, typically mammalian host cell systems, for mammalian protein production. One feature of such systems is that the protein produced has a structure most like that of the natural protein species and purification often is easier since the protein can be secreted into the culture medium in a biologically active form.

One of the most efficient mammalian cell expression systems is based on Vaccinia virus. The main problem with this system, however, is that it uses recombinant viruses that express the heterologous gene upon infection. Thus there is no control over the virus once it has been release.

Recently researchers have started to explore the use of positive strand RNA viruses such as Semliki Forest Virus (SFV), Sindbis (SIN) virus, and poliovirus, as vectors for expression of heterologous genes in *vitro* and in *vivo*. The success of these expression systems has been mainly based on each virus's ability to produce high titer stocks of "pseudo" infectious particles containing recombinant replicon RNA packaged by structural proteins. In commercially available Semliki Forest virus (SFV) and Sindbis virus expression systems this is achieved by co-transfection of replicon RNA with defective helper RNA(s) expressing structural genes, but lacking the packaging signal. Replicon RNA expression provides the enzymes for RNA replication and transcription of both RNA's, whereas the helper RNA supports the production of structural proteins for packaging of replicon RNA via expression of its subgenomic region. The main problem with these expression systems is that the viruses used in the expression system are cytopathic and often compete out the host protein synthesis. Another major disadvantage of these systems includes possible contamination with infectious particles containing packaged full-length genomic RNA (in other words, infectious virus) due to the high probability of recombination between replicon and helper RNAs.

The present invention seeks to provide an improved expression system that at least ameliorates some of the problems associated with prior art systems.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or group of integers, but not the exclusion of any other integer or group of integers including method steps.

Thus, the present invention provides a heterologous gene expression system comprising:

a) a self-replicating expression vector derived from flavivirus RNA, which vector lacks at least a structural gene and is adapted to receive at least a genetic sequence heterologous to the flavivirus genome; and

b) at least a second vector which is capable of expressing structural Flavivirus proteins.

Preferably, the second vector is engineered to prevent recombination with the self-replicating expression vector. More preferably, the second vector is heterologous in origin to the origin of the self-replicating expression vector. By using a viral RNA which is heterologous to flavivirus RNA for expression of structural genes essential for packaging, it is possible to minimise any possibility of contamination with infectious flavivirus. For example, if a Kunjin (KUN) replicon is used as the self-replicating expression vector, then the second vector might be derived from an alphavirus (by way of example) such as SFV or SIN. In a highly preferred form of the invention the self-replicating expression vector is derived from KUN while the second vector is derived from SFV to take account of the impossible recombination between KUN RNA and SFV RNA.

According to the present invention, the self-replicating expression vector (replicon) of flavivirus origin is adapted to receive at least a genetic sequence which is heterologous to the flavivirus genome. Preferably, where a plurality of heterologous genetic sequences are employed in the replicon at least one of the sequences should encode an amino acid sequences that needs to be amplified. For example, the replicon might contain the gene sequence of a secreted protein or peptide sequence.

The present invention is described in terms of inserting heterologous genetic sequences into the replicon, the use of the term "genetic sequence" is intended to include, within its broad context, parts of genes and gene sequences. For example, heterologous gene sequences may encode sequences appropriate of

promoting replication or expression. Alternatively or in addition, the sequence may encode proteins which need to be expressed.

Although the invention is described as a means for producing proteins, the term "protein" should be understood to include within its scope parts of proteins (eg peptide and polypeptide sequences). For example, the replicon might be used to express an amino acid sequence which when secreted from a cell or is expressed by a cell is capable of eliciting an immunogenic response.

In use, the replicon is introduced into a host cell where gene expression and hence protein production take place. Because the vector is capable of self-replication, multiple copies of the replicon will also be generated. This leads to an exponential increase in the number of replicons in the host cell as well as an exponential increase in the amount of protein that is produced.

Upon introduction of the second vector, containing the structural genes necessary to produce virus particles, structural proteins are produced. These proteins encapsulate the replicon therein forming a "pseudo" recombinant virus that is only capable of producing heterologous protein inside another cell. The pseudo-virus can not however replicate to produce new viral particles because the genes necessary for the production of the structural proteins are not provided in the replicon. (PseudoVirus stock will only be produced when co-transfection of the replicon and the vector bearing the structural genes occurs.

Some advantages associated with the use of the present invention are provided below.

(1) The flavivirus expression system has relatively high level of protein expression in eukaryotic cell lines.

(2) The flavivirus expression system is capable of expressing proteins in a wide variety of mammalian cell lines and cell types.

(3) The replicons used in the flavivirus expression system produce a persistent non-cytopathic infection in host cells. There are no observable effects on the host's translation process. This feature of flavivirus replicons also allows selection of stable cell lines

continuously expressing other genes using a replicon vector expressing Neo gene.

(4) The flavivirus expression system is an RNA system that does not permit integration of viral genomic material into a host's genomic sequence.

The replication of flaviviruses is quite different from other viruses. For example, flaviviruses differ from alphaviruses (such as SFV and SIN) by their genome structure (structural genes situated at the 5' end of the genome) and by the absence of synthesis of subgenomic RNA. Furthermore, there are no data to date on packaging of flavivirus RNA.

Substantial progress in the development of mammalian cell expression systems has been made in the last decade, and many aspects of these systems' features are well characterised. A detailed review of the state of the art of the production of foreign proteins in mammalian cells, including useful cell lines, protein expression-promoting sequences, marker genes, and gene amplification methods, is disclosed in Bendig, M., (1988) Genetic Engineering 7: 91-127.

It will be appreciated that any replicon derived from flavivirus RNA, which is lacking at least a structural gene and which is adapted to receive at least a genetic sequence heterologous to the flavivirus genome may be employed in the present invention. Optimal flavivirus replicon design for transfection into eukaryotic cells might include such sequences as: sequences to promote expression of the heterologous gene of interest, including appropriate transcription initiation, termination, and enhancer sequences; as well as sequences that enhance translation efficiency, such as the Kozak consensus sequence. To prepare stable cell lines capable of persistently expressing replicon RNAs, the described vectors may also be constructed in selectable form by inserting an IRES-Neo cassette into the 3'UTR.

In a highly preferred form of the invention the replicon used in the invention should be adapted to include at least, about the first 150 nucleotides of a flavivirus genome, at least about the last 60 nucleotides of E protein, substantially

all of the nonstructural region, and part or all of the 3'UTR. Replication of a flavivirus genome is dependent on the genes in the nonstructural region of the genome being present during transcription and translation. Any modification that is made to the nonstructural region must not interfere with the functional activity of the genes within the nonstructural region of the flavivirus genome. In one embodiment of the invention, the replicon includes the first 157 nucleotides of the KUN genome, the last 66 nucleotides of E protein, all of the nonstructural region, and all of the 3'UTR.

Insertion of a heterologous genetic sequence, which encodes a protein, into the described replicon may be achieved at any point in the replicon that does not effect processing of flavivirus proteins. For example, heterologous genes may be inserted into the 3' UTR of the flavivirus replicon or within the locality of deleted structural genes. Preferably, heterologous genes are inserted in place of deleted structural genes since such insertions generally produce higher levels of expression and generally do not affect replication efficiency of the replicon. If heterologous genes are inserted into the 3'UTR they should be preceded by a IRES sequence. Most preferably, heterologous genes are inserted into the locality where the structural genes were deleted from. In a highly preferred form of the invention, the 3' UTR is used only for insertion of IRES-Neo sequences. Such an insertion would allow the generation of stable cell lines persistently expressing foreign genes via antibiotic (eg Geneticin) selection.

While the heterologous genetic sequence encoding a protein may be placed under the control of the host's own regulatory machinery in the replicon, it will be appreciated that it may be associated with one or more alternate regulatory elements capable of promoting its expression. Such elements will be well known to those of ordinary skill in this field.

The replicons described herein can be engineered to express multiple heterologous sequences allowing co-expression of several proteins. Multiple expression of proteins would be useful for example where the replicon expresses a plurality of antigens (ie. protective antigens) along with cytokines or other

immunomodulators to enhance the generation of an immune response. Such a replicon might be particularly useful for example in the production of various proteins at the same time or in gene therapy applications.

Desirably, the second vector which contains the flavivirus structural genes is engineered to prevent recombination with the self-replicating expression vector. One means for achieving this end is to prepare the second vector from genetic material which is heterologous in origin to the origin of the self-replicating expression vector. For example, the second vector might be prepared from SFV when the replicon is prepared from KUN virus.

To optimise expression of the flavivirus structural genes, the second vector might include such sequences as: sequences to promote expression of the genes of interest, including appropriate transcription initiation, termination, and enhancer sequences; as well as sequences that enhance translation efficiency, such as the Kozak consensus sequence. Preferably, the second vector contains separate regulatory elements associated with each of the different structural genes expressed by the vector. Most preferably, the flavivirus C gene and the prME genes are placed under the control of separate regulatory elements in the vector.

The processing of flavivirus structural proteins during virus replication in cells is rather complex and requires a number of post-translational cleavages by host and viral proteases. Numerous *in vitro* and *in vivo* studies on processing of the C-prM region have established two cleavage events: cleavage at a dibasic cleavage site by viral NS2B-NS3 protease generating the carboxy terminus of mature virion C protein, which appears to be a prerequisite for the efficient cleavage at the NH_2 terminus of prM by cellular signalase. While viral proteases are expressed by the replicon during expression of the genes forming the nonstructural region of a flavivirus, it will be appreciated that the second vector may also be adapted to include genes encoding viral NS2B-NS3 protease.

Host cell lines contemplated to be useful in the method of the invention include any eukaryotic cell lines that can be immortalised, ie., are viable for multiple passages, (eg., greater than 50 generations), without significant reduction in

growth rate or protein production. Useful cell line should also be easy to transfect, be capable of stably maintaining foreign RNA with an unrearranged sequence, and have the necessary cellular components for efficient transcription, translation, post-translation modification, and secretion of the protein. Currently preferred cells are those having simple media component requirements, and which can be adapted for suspension culturing. Most preferred are mammalian cell lines that can be adapted to growth in low serum or serum-free medium. Representative host cell lines include BHK (baby hamster kidney), VERO, C6-36, COS, CHO (Chinese hamster ovary), myeloma, HeLa, fibroblast, embryonic and various tissue cells, eg., kidney, liver, lung and the like and the like. Useful cells can be obtained from the American Type Culture Collection (ATCC), Rockville, Md. or from the European Collection of Animal Cell Cultures, Porton Down, Salisbury SP40JG, U.K.

With respect to the transfection process used in the practice of the invention, all means for introducing nucleic acids into a cell are contemplated including, without limitation, CaPO.sub.4 co-precipitation, electroporation, DEAE-dextran mediated uptake, protoplast fusion, microinjection and lipofusion. Moreover, the invention contemplates either simultaneous or sequential transfection of the host cell with vectors containing the RNA sequences to be integrated into the genome. In one preferred embodiment, host cells are simultaneously transfected with at least two unlinked vectors, one of which contains the heterologous gene, and the other of which contains the structural genes.

Further features of the present invention are more fully described in the following Figures and Examples. In the figures:

Figure 1 illustrates a schematic representation of the recombinant KUN constructs of AKUN, FLSD and FLSDX.

Figure 2 illustrates a schematic representation of the recombinant SFV constructs. The solid line in all constructs represents the segment of the SFV replicon genome flanking the multiple cloning site, open boxes show the inserted KUN structural genes C, prM, and E as indicated, 26S shows

the position of the subgenomic SFV promoter, the filled and partially filled boxes in the KUN prM and E genes represent hydrophobic signal and anchor sequences, respectively. Capital letters in the nucleotide sequences show authentic KUN nucleotides, small letters show nucleotides derived from the pSFV1 vector or encoded in the primers used for PCR amplification of KUN genes. Bold and italicised letters show initiation (ATG) and termination (taa, tag) codons. Numbers with arrows represent amino acid positions in the KUN polyprotein. Msc, Sma, Spe, Bam, and Bgl represent specific restriction sites. Asterisks indicate that these restriction sites were destroyed during the cloning procedure.

Figure 3 illustrates expression of KUN C protein by recombinant SFV-C replicon. A) Immunofluorescence analysis of BHK21 cells at 18h after transfection with SFV-C RNA (SFV-C, panels 1, 3, and 5) using KUN anti-C antibodies (see Materials and Methods). SFV1 (panels 2, 4, and 6) represents IF of cells transfected with the control SFV1 RNA prepared from pSFV1 vector. Cells in panels 1 and 2 were photographed at lower magnification then in panels 3 to 6. Ace is an abbreviation for acetone fixation, F+Me is an abbreviation for formaldehyde-methanol fixation. B) Metabolic labelling with ^{35}S-methionine/cysteine and radioimmunoprecipitation analysis with antibodies to C protein (+anti-C) of SFV-C and SFV1 transfected BHK21 cells. BHK21 cells in 60mm culture dishes at 18h after transfection were continuously labelled with 50 μCi/ml of ^{35}S-methionine/cysteine for 4h. Labelled cell lysates and radioimmunoprecipitates were prepared and samples were electrophoresed in a 15% polyacrylamide gel. Sample volumes were 1 μl of 500 μl in SFV-C, 0.5 μl of 300 μl in SFV1, 10 μl of 30 μl radioimmunoprecipitate from 160 μl of both SFV-C and SFV1 (+anti-C) cell lysates. Dots indicate the location of KUN proteins NS5, NS3, E, NS4B, prM, NS2A, C, and NS4A/NS2B (from top to bottom) in the radiolabeled KUN infected cell lysate. The arrow shows position of KUN C protein. Numbers represent molecular weights of low range pre-stained Bio-Rad

protein standards. This and following figures were prepared by scanning all the original data (slides, autoradiograms, etc.) on the Arcus II scanner (Agfa) using FotoLook software (Agfa) for Macintosh at 150 lpi resolution, followed by assembling of the montages using Microsoft PowerPoint 97 software and printing on Epson Stylus Color 800 printer at 720-1440 dpi resolution using Epson photo quality ink jet paper.

Figure 4 illustrates expression of KUN prME genes by recombinant SFV replicon. A) IF analysis of SFV-prME and SFV1 transfected BHK21 cells at 18h after transfection using KUN monoclonal anti-E antibodies. (B) and (C) show the results of pulse-chase labelling and radioimmunoprecipitation analysis with KUN monoclonal anti-E antibodies, respectively, of SFV-prME transfected BHK21 cells, where CF (culture fluid) and C (cells) represent samples collected during chase periods. Lanes 1 to 9 in (B) and (C) represent the same samples either directly electrophoresed in 12.5% SDS-polyacrylamide gel (B), or radioimmunoprecipitated with anti-E antibodies followed by electrophoresis in a 12.5% SDS-polyacrylamide gel (C). Lanes 2 and 9 show samples collected after a 4h-chase period from culture fluid and cells, respectively, after transfection with the control SFV1 RNA. Lanes 3, 4, and 5 show culture fluid samples collected at 1h, 4h, and 6h of chase periods, respectively, and lanes 6, 7, and 8 show the corresponding chase samples from the cells. In (B) 10μl of total 700 μl of culture fluid and 5 μl of total 300 μl of cell lysates samples were used for electrophoresis. In (C) 10 μl of total 30 μl of immunoprecipitate prepared either from 150 μl of the cell lysate or from 350 μl of the culture fluid were used for electrophoresis. The exposure time of the dried gel for cell lysates was 1day, and 5 days for culture fluids. Dots in lane 1 of (B) and (C) indicate KUN proteins in the radiolabeled KUN cell lysates, as in Fig. 2B. Numbers represent molecular weights in the low range pre-stained Bio-Rad protein standards.

Figure 5 illustrates expression of all three KUN structural proteins by the recombinant SFV-prME-C replicon. A) Double IF analysis of the same field

in BHK21 cells at 18h after transfection with SFV-prME RNA using KUN anti-C (panel 1) and anti-E (panel 2) antibodies, with Texas Red (TR) and FITC conjugated secondary antibodies, respectively. In (B) and (C), cells at 18h after transfection with SFV-prME-C RNA were pulsed with ^{35}S-methionine/cysteine for 1h; subsequently, 300 μl (from total of 600μl) of cell lysates ("C" in [B] and in [C]) and 1ml (from total of 2ml) of culture fluids ("CF" in [B]) collected at different chase intervals (1h, 6h, and 9h), were immunoprecipitated either with KUN monoclonal anti-E antibodies (B), or with KUN anti-C antibodies (C). Ten μl (from total of 30μl) of immunoprecipitated samples were electrophoresed in 12.5% (B) and 15% (C) SDS-polyacrylamide gels. Dots in (B) indicate KUN proteins in the labelled KUN cell lysates as in Fig. 2B. Dots in (C) represent KUN proteins prM, NS2A, C, and NS4A/NS2B (from top to bottom) in the radiolabeled KUN infected cell lysate. Numbers represent molecular weights of the low range pre-stained Bio-Rad protein standards.

Figure 6 illustrates packaging of KUN replicon RNA by KUN structural proteins expressed from the recombinant SFV replicons. (A) IF analysis with KUN anti-NS3 antibodies of BHK21 cells infected with the culture fluid collected from BHK21 cells at 26h after transfection first with C20DXrep RNA and 26h later with SFV-prME-C RNA (panel 1), or with SFV-prME and SFV-C RNAs (panel 2), or with SFV-prME RNA (panel 3). (B) and (C) show Northern blot analysis of RNAs isolated from BHK21 cells infected as described in (A), using labelled KUN-specific (B) and SFV-specific (C) cDNA probes. Lane 1 in (B) and lane 2 in (C) correspond to the cells in panel 1 in (A). Lane 2 in (B) and lane 3 in (C) correspond to the cells in panel 2 in (A). Lane 1 in (C) represents in vitro synthesized SFV-prME-C RNA. Arrows in (B) and (C) indicate the positions of RNAs of about 8.8 kb for KUN replicon RNA and about 10.8 kb for SFV-prME-C RNA determined relative to migration in the same gel of ethidium bromide-stained λ DNA digested with BstEII (New England Biolab).

Figure 7 illustrates optimisation of conditions for packaging of KUN replicon RNA. Northern blot analysis of BHK21 cells infected with filtered and RNase-treated culture fluid samples. In (A), samples were collected at a fixed time (24h) after second transfection (with SFV-prME-C RNA) and using different time intervals as shown between transfections of C20DXrep and SFV-prME-C RNAs. In (B), samples were collected at different times as shown after the second transfection (with SFV-prME-C RNA) which occurs at a fixed time (30h) after the first transfection (with C20DXrep RNA). The probe in both (A) and (B) was a radiolabeled cDNA fragment representing the last 761 nucleotides of the KUN genome. Titers in (A) shown under the lanes in the Northern blot represent the amounts of infectious units (IU) contained in the corresponding samples of culture fluids used for infections and determined by IF analysis with anti-NS3 antibodies and counting of IF positive cells.

Figure 8 illustrates characterisation of infectious particles. (A) Inhibition of infection with encapsidated particles, released from cells transfected sequentially with C20DXrep and SFV-prME-C RNAs (as in Fig. 6), by incubation with KUN anti-E monoclonal antibodies. Panel 1 represents IF with anti-NS3 antibodies of cells infected with culture fluid collected after the transfections and incubated with anti-E monoclonal antibodies for 1h at 37°C. Panel 2 represents IF with anti-NS3 antibodies of cells infected with the same sample of culture fluid incubated under similar conditions in the absence of anti-E antibodies. (B) shows IF analysis with anti-N3 antibodies of cells infected with equal proportions of resuspended pellet (panel 1; 2μl from 50μl of total volume) or supernatant fluid (panel 2; 200μl from 5ml of total volume) from the culture fluid collected from cells transfected with C20DXrep and SFV-prME-C RNAs and subjected to ultracentrifugation. (C) Radioimmunoprecipitation analysis with anti-E antibodies of culture fluids from cells transfected with SFV-prME-C RNA (lane 2), sequentially transfected with C20DXrep and SFV-prME-C RNAs (lane 1), and infected with KUN virus (lane 3). Dots show faint bands corresponding to C and

prM visible (in the original autoradiogram) in lane 1, but only a faint band for prM in lane 2. (D) RT-PCR analysis with KUN-specific primers of RNAs extracted from the anti-E-immunoprecipitates of culture fluid samples collected after transfection sequentially with C20DXrep and SFV-prME-C RNAs (lane 2), or after transfection only with SFV-prME-C RNA (lane 3), or after infection with KUN virus (lane 4). Lane 1 represents PhiX174 RF DNA digested with HaeIII (New England Biolab).

It is to be understood that the following Examples are included solely for the purposes of exemplifying the invention, and should not be understood in any way as a restriction on the broad description as set out above.

EXAMPLES

Cells. BHK21 cells were grown in Dulbecco's modification of minimal essential medium (Gibco BRL) supplemented with 10% foetal bovine serum at 37°C in a CO_2 incubator.

Example 1

Construction of the replicons and vectors.

(i) C20rep: All deletion constructs were prepared from the cDNA clones used in the construction of the plasmid pAKUN for generation of the infectious KUN RNA (Khromykh and Westaway, 1994) by PCR-directed mutagenesis using appropriate primers and conventional cloning. dME cDNA and its derivatives were deleted from nucleotides 417 to 2404, which represent loss of the signal sequence at the carboxy terminus of C now reduced to 107 amino acids, deletion of prM and E, with the open reading frame resumed at codon 479 in E, preceding the signal sequence for NS1. C20 rep and C2rep cDNAs represent progressive in frame deletions in coding sequence of C leaving only 20 or 2 amino acids of C, respectively, with the open reading frame continued at codon 479 in E, as in dME.

(ii) FLSDX: All RT reactions were performed with Superscript II RNase H-reverse transcriptase (Gibco BRL) essentially as described by the manufacturer

using100-200ng of purified KUN virion RNA, or 1μg of total cell RNA and appropriate primers. PCR amplification after RT of a 6895bp DNA fragment was performed with the Expand High Fidelity PCR kit (Boehringer Mannheim) using 1/25 volume of RT reaction and appropriate primers as follows. The PCR reaction mixture (50 μl) containing all necessary cornponents except the enzyme mixture (3 parts of Taq polymerasse and 1 part of Pwo polymerase) was preheated at 95°C for 5 min, then the enzyme mixture was added and the following cycles were performed: 10 cycles of 95°C for 15sec and 72°C for 6min, followed by 6 cycles of 95°C for 15sec and 72°C for 6min with an automatic increase of extension time at 72°C for 20sec in each following cycle. All PCR reactions with Pfu DNA polymerasse (Stratagene) were performed essentially as described by the manufacturer using 1/25-1/10 volumes of RT reactions and appropriate primers.

All plasmids shown in Fig. 1A were obtained from the previously described stable KUN full-length cDNA clone pAKUN (Khromykh and Westaway, 1994) by substitution of the original cDNA fragments with those obtained by RT and PCR amplification of purified KUN RNA using existing unique restriction sites which were also incorporated into the primers for PCR amplification.

Initially the SacII-DraIII (~7kb) fragment in pAKUN clone (Fig. 1) was replaced with the fragment amplified using Expand High Fidelity PCR kit from the cDNA obtained by reverse transcription of purified KUN virion RNA using appropriate primers. Substitution of this fragment resulted in the restoration of the previously mutated BamHI site in NS2A gene of pAKUN plasmid and appearance of a silent mutation by introducing a new unique AflII site incorporated into the primer used for PCR amplification (Fig. 1). RNAs transcribed from several resulting cDNA clones (FLSD) were tested for efficiency of transfection/replication after electroporation into BHK21 cells. From nineteen RNAs examined, two were highly efficient (~60-80% cells positive by IF with anti-NS3 antibodies at 21h after transfection of ~5 μg RNA), five were moderately efficient (5-10%), two were poorly efficient (0.5-1%), and ten were not infectious at all. For comparison, about 2-5% cells were positive at 21h after transfection with ~10 μg of transcribed

AKUN RNA. When cells transfected with one of the efficient FLSD RNAs were incubated for another 25h (total 46h), virtually all cells were positive by IF and most of the cells appeared to show severe cytopathic effects indicating quick spread of the replicating virus. In contrast, the proportion of positive cells remained almost unchanged, and no apparent signs of virus spread (foci) were detected at 46h after transfection of AKUN RNA. These results indicate that some unidentified mutations inhibiting RNA replication (21h results) and virus spread (46h results) were present in the original pAKUN cDNA. Further determination of the specific infectivity by plaque assay showed that FLSD RNA had a specific infectivity of ~2/103 PFU per 1 μg, compared to only 1-5 PFU per 10 μg for AKUN RNA (Fig.1). This FLSD cDNA clone producing RNA with relatively high specific infectivity was used in further reconstruction experiments. Because the specific infectivity of FLSD RNA was still lower than that of KUN virion RNA (Fig. 1), the rest of the genome was replaced in an attempt to improve efficiency even further. Taking in account the great variability in specific infectivity of FLSD cDNA clones constructed with a substituted DNA fragment obtained using an Expand High Fidelity PCR kit, Pfu DNA polymerase (Stratagene) was used with the highest replication fidelity amongst thermostable DNA polymerases. Replacement in FLSD cDNA of a 2645 nts fragment covering most of the NS5 gene and the 3'UTR produced FLSDX and resulted in a further 5- to 10-fold improvement of the specific infectivity.

(iii) C20DXrep. KUN replicon cDNA construct C20DXrep was constructed from described above C20rep by replacing an *Sph*I^{2467}- *Xho*I^{11021} fragment representing the sequence coding for the entire nonstructural region and the 3'UTR with the corresponding fragment from a stable full-length KUN cDNA clone FLSDX. Transfection of BHK cells with 5-10 μg of C20DXrep RNA resulted in detection of ~80% replicon-positive cells compare to only ~10% positive after transfection with the same amount of C20rep RNA.

(iv) SFV-C. An SFV replicon construct expressing KUN core (C) gene was obtained by cloning of the *Bgl*II-*Bam*HI fragment, representing the sequence of the last 7 nucleotides of the KUN 5'UTR and the sequence coding for the first 107

of the 123 amino acids of KUN C protein, from the plasmid pCINeoC107 (Khromykh, A. A. and E. G. Westaway. 1996). RNA binding properties of core protein of the flavivirus Kunjin. Arch. Virol. 141: 685-699) into the *Bam*HI site of the SFV replicon expression vector pSFV1 (Gibco BRL; Fig. 2).

(v) SFV-prME. KUN prME sequence was PCR amplified from another highly efficient full-length KUN cDNA clone FLBSDX modified from FLSDX (which will be described elsewhere), using appropriate primers with incorporated *Bgl*II sites. The amplified fragment was digested with *Bgl*II and cloned into the *Bam*HI site of the SFV replicon expression vector pSFV1 to obtain the SFV-prME construct (Fig. 2).

(vi) SFV-prME-C. SFV replicon construct expressing both KUN prME and KUN C genes was obtained by cloning a *Msc*I-*Spe*I fragment from the SFV-C plasmid containing the SFV 26S subgenomic promoter, KUN C sequence and SFV 3'UTR into the SFV-prME vector digested with *Sma*I and *Spe*I (Fig. 2). Thus the final double subgenomic construct SFV-prME-C should produce SFV replicon RNA which upon transfection into BHK cells will direct synthesis of two subgenomic RNAs expressing KUN prME and KUN C genes.

RNA transcription and transfection. RNA transcripts were prepared from C20DXrep plasmid DNA linearized with *Xho*I, and from SFV plasmids linearised with *Spe*I using SP6 RNA polymerase. Electroporation of RNAs into BHK21 cells was performed. Briefly, 10-20 μg of in vitro transcribed RNAs were electroporated into 2×10^6 BHK21 cells in 400 μl in a 0.2-cm cuvette (Bio-Rad) using the Bio-Rad Gene Pulser apparatus.

Immunofluorescence analysis. Replication of KUN replicon RNA C20DXrep after initial electroporation, and after infection of BHK cells in packaging experiments, was monitored by immunofluorescence (IF) analysis with antibodies to KUN NS3 protein. Expression of KUN E protein after electroporation with SFV-prME and SFV-prME-C RNAs was detected by IF with a cocktail of mouse monoclonal antibodies to KUN E protein. These antibodies designated 3.91D, 10A1, and 3.67G were generously provided by Roy Hall, University of

Queensland, Brisbane, Australia. All three antibodies were mixed in equal amounts and a 1/10 dilution of this mixture was used in IF analysis. Expression and nuclear localisation of KUN C protein after electroporation with SFV-C and SFV-prME-C RNAs was monitored by IF analysis with rabbit polyclonal antibodies to KUN C protein .

Metabolic labelling and radioimmunoprecipitation analysis. Metabolic labelling of electroporated BHK cells with ^{35}S-methionine/cysteine was performed as described in the SFV Gene Expression System Manual (Gibco BRL) with some minor modifications. Briefly, at 18 h after the electroporation with SFV RNAs, cells were pulse labelled with ^{35}S-methionine/cysteine for 4h, or for 1-2h followed by different periods of incubation (chase) in medium with an excess of unlabelled methionine/cysteine. Cell culture fluid was collected and samples were either electrophoresed directly in polyacrylamide gels or used for radioimmunoprecipitation (RIP) analysis with anti-E and anti-C antibodies. Labelled cells were lysed in buffer containing 1% Nonidet P40 (NP40), 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 2mM EDTA, the nuclei removed by low speed centrifugation and the lysate supernatant was either electrophoresed directly in a SDS-polyacrylamide gel or used for RIP with anti-E and anti-C antibodies.

For RIP analysis, labelled cell culture fluid and cell lysates were mixed with 1/20 volume of the cocktail of three anti-E monoclonal antibodies (see above) or with rabbit anti-C antibodies, and incubated overnight at 4°C with constant rotation in microcentrifuge tubes. Protein A-Sepharose beads were then added to a final concentration of about 1%, and incubation was continued for another 1h at 4°C. After three washes with RIPA buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP40; 0.5% deoxycholic acid sodium salt [DOC]; 0.1% sodium dodecyl sulfate [SDS]) and one wash with phosphate buffered saline (PBS), beads were resuspended in the SDS-gel sample buffer, boiled for 5 min and subjected to electrophoresis in an SDS-polyacrylamide gel. After electrophoresis gels were dried and exposed to X-ray film.

Northern blot hybridisation. Five μg total RNA, isolated using Trizol reagent (Gibco BRL) from BHK21 cells infected with culture fluid collected from cells doubly transfected with C20DXrep RNA and SFV RNAs expressing KUN structural proteins, was electrophoresed for Northern blotting. The hybridisation probes were [^{32}P]-labelled cDNA fragments representing the 3'-terminal 761 nucleotides of the KUN genome including the 3'UTR region (see Fig. 6B and Fig. 7), and 446 nucleotides of the SFV NSP2 region (see Fig. 6C).

Expression of KUN C gene by the recombinant SFV-C replicon. For the expression of KUN C gene in the pSFV1 vector the *Bgl*II-*Bam*HI fragment from plasmid pCINeoC107 was subcloned into the *Bam*HI site of pSFV1 (Fig. 2). This fragment represents the sequence coding for the first 107 amino acids of KUN C protein, equivalent to the mature form of C, from which the carboxy terminal hydrophobic sequence has been removed. The SFV-C construct also contains a native KUN initiation codon with an extra 7 nucleotides of the KUN 5'UTR derived from the pCINeoC107 plasmid and four extra codons at the carboxy-terminus derived from the SFV vector sequence (Fig. 2).

Electroporation of SFV-C RNA into BHK21 cells resulted in expression of KUN C protein in almost 100% of cells as judged by IF with antibodies to KUN C protein (Fig.3A, panel 1). KUN C protein expressed in SFV-C RNA transfected cells was localised in the cytoplasm (Fig. 3A, panel 3; acetone fixation) and also in the nuclei (Fig. 3A, panel 5; formaldehyde-methanol fixation). Because of difficulties in identification of KUN C protein in radiolabeled lysates of SFV-C transfected cells (Fig 3B), immunoprecipitation of the radiolabelled lysates with anti-C antibodies was carried out. A labelled band coincident in migration with KUN C protein was apparent in the lysates of SFV-C but not in those of SFV1 transfected cells (compare SFV-C and SFV1 in Fig. 3B).

Expression of KUN prME genes by the recombinant SFV-prME replicon. The full-length prME sequence plus the preceding signal sequence in our SFV-prME construct (see Fig. 2) was included in the replicon. As a source of cDNA for prME genes, full-length KUN cDNA clone FLBSDX were used. An initiation and a

termination codon, as well as *Bgl*II sites for conventional cloning, were incorporated into the primers for PCR amplification (see Fig. 2). To minimise the amount of possible mismatches which could occur during PCR amplification high fidelity Pfu DNA polymerase (Stratagene) was used in all our PCR reactions.

When SFV-prME RNA was electroporated into BHK21 cells, nearly 100% of cells were found to be positive in IF analysis with monoclonal antibodies to KUN E protein at 12-18h after electroporation (Fig. 4A, panel 1). To confirm expression of KUN prM and E proteins in transfected cells and to detect secretion of prME into the tissue culture fluid transfected cells were labelled with ^{35}S-methionine/cysteine for 1h, followed by incubation for increasing chase periods. A strongly labelled band corresponding to KUN E protein was apparent in both culture fluid and cell lysates of SFV-prME transfected cells at all times (see culture fluid and cells in Fig. 4B). A labelled band corresponding to KUN prM protein was detected only in cell lysates (cells in Fig. 4B). A labelled band corresponding in migration to the predicted molecular weight of KUN pr protein was detected in the culture fluid only of transfected cells (culture fluid in Fig. 4B).

An apparent increase in the intensity of labelling of E and possibly pr proteins in the culture fluid (Fig. 4B, culture fluid) and a concomitant decrease in the intensity of labelling of E and prM proteins in the cell lysates (Fig. 4B, cells) were observed during the chase period. The efficiency of the secretion of E and pr proteins was low, since the lanes showing labelled culture fluid were exposed to X-ray film for about 5 times longer than the lanes showing cell lysates (see legend to Fig. 4).

When samples from the pulse-chase labelling experiment with SFV-prME replicon were immunoprecipitated with KUN anti-E monoclonal antibodies, E and prM were coprecipitated from the transfected cell lysates (Fig. 4C, lanes 6-9). E protein (Fig. 4C, lanes 3-5) and in some experiments trace amounts of prM protein (results not shown) were precipitated also from culture fluid of transfected cells. Because of its low molecular weight, M protein probably ran off the gel during electrophoresis and therefore could not be detected. A gradual increase in

the amount of immunoprecipitated labelled E protein in the culture fluid of transfected cells was observed throughout the chase period (Fig. 4C, lanes 3-5), confirming the ongoing secretion of E protein. An absence of correlation between the increase of immunoprecipitated labelled E protein in the culture fluid, and an expected decrease of labelled E and prM proteins immunoprecipitated from the cell lysate (compare lanes 3-5 in Fig. 4C with the corresponding culture fluid lanes in Fig. 4B, and lanes 6-9 in Fig. 4C with the corresponding cell lanes in Fig. 4B), can probably be explained by inadequate amounts of antibodies used for immunoprecipitation of a large excess of expressed proteins retained in the cells during the relatively short chase periods. Taken together, results of the direct pulse-chase labelling and RIP analyses confirmed both the correct processing of prME polyprotein in cells and the secretion of E, and possibly pr and M proteins, into the culture fluid after transfection of SFV-prME RNA into BHK21 cells.

Expression of all three KUN structural proteins by the recombinant SFV-prME-C replicon. Although KUN replicon was packaged using transfection with two SFV RNAs expressing prME and C genes separately (see results in the next example), the efficiency of packaging was low. To increase the efficiency of packaging and to simplify the procedure a single SFV replicon construct was prepared expressing prME genes and C gene simultaneously. Because of the difficulties experienced with cloning of the entire C-prM-E region into the pSFV1 vector (see the first section of the Results) and also in order to avoid possible uncertainty regarding cleavage at the carboxy terminus of C in the absence of flavivirus protease NS2B-NS3, an SFV replicon expressing prME and C genes under the control of two separate 26S promoters was prepared (see SFV-prME-C in Fig. 2).

IF analysis of SFV-prME-C electroporated BHK cells with anti-E and anti-C antibodies showed expression of both E and C proteins in nearly 100% of cells by 18h after transfection (results not shown). Both E and C proteins were expressed in the same cell (compare dual labelling by anti-C and anti-E antibodies in Fig. 5A). When transfected cells were pulse-chased with ^{35}S-methionine/cysteine and the lysates were immunoprecipitated with KUN anti-E monoclonal antibodies,

both E and prM proteins were coprecipitated, as was observed after transfection of SFV-prME RNA (compare Fig. 4B and Fig. 5B). A gradual increase of immunoprecipitated labelled E protein in culture fluids, and a corresponding decrease of immunoprecipitated labelled E and prM proteins in the cell lysates were observed during the chase period (Fig. 5B). Immunoprecipitation of the labelled cell lysates with anti-C antibodies confirmed expression of C protein in transfected cells and showed a gradual decrease of the amount of precipitated C during the chase period (Fig. 5C). The results of RIP analysis of culture fluid, not treated with detergents, using anti-C antibodies were negative (results not shown), indicating that no free C protein was secreted into culture fluid of SFV-prME-C transfected cells. In a later experiment (see Fig. 8C, lane 2), particles secreted from cells transfected only with SFV-prME-C RNA were purified and precipitated with anti-E antibodies; again no secreted C was detected.

Overall, the immunofluorescence and labelling patterns in cells transfected with SFV-prME-C RNA were very similar to those observed in cells transfected with two different RNAs expressing prME and C proteins separately (compare Fig. 5 with Fig. 3 and Fig. 4), suggesting proper processing and maturation of all three KUN structural proteins when expressed from the recombinant SFV replicon.

Example 2

Preparation of encapsidated particles and determination of their titer.

For all infections with encapsidated particles, cell culture fluid was filtered through a 0.45 μm filter (Sartorius AG, Gottingen, Germany) and treated with RNase A (20μg per ml) for 0.5h at room temperature (followed by 1.5h incubation at 37°C during infection of cells). To prepare partially purified particles, filtered and RNase A treated culture fluids from transfected cells were clarified by centrifugation at 16,000g in the microcentrifuge for 15 min at 4°C, and the particles were pelleted from the resulting supernatant fluid by ultracentrifugation at 40,000 rpm for 2h at 4°C in the AH650 rotor of the Sorvall OTD55B centrifuge. The pellets were resuspended in 50 μl PBS supplemented with RNAse A (20μg per ml), left to dissolve overnight at 4°C, and then used for infection of BHK21

cells or for RT-PCR analysis. To determine the titer of encapsidated particles, BHK21 cells on 1.3 cm^2 coverslips were infected with 100-200 µl of serial 10-fold dilutions of cell culture fluid or of pelleted material for 1.5h at 37°C. The fluid was then replaced with 1ml of DMEM medium supplemented with 2% FBS; cells were incubated for 24h at 37°C in the CO_2 incubator and then subjected to IF analysis with anti-NS3 antibodies as described above. The infectious titer of packaged particles was calculated using the following formula:

$$\text{Titer (IU) per } 2\times10^6 \text{ of initially transfected cells} = N\times(SW{:}SIA)\times10^n\times(V{:}\ VI),$$

where N is the average number of anti-NS3 positive cells in the image area, calculated from 5 image areas in different parts of the coverslip; SW is the surface of the well in a 24-well plate (200 mm^2); SIA is the surface of the image area (1.25 mm^2 using defined magnification parameters, calculated according to the manual for the Wild MPS46/52 photoautomat [Wild Leitz, Heerburg, Germany]); V is the total volume of the culture fluid (usually 3-5 ml per 60 mm dish) collected from the population of 2×10^6 initially electroporated BHK21 cells; VI is the volume used for infection of the cover slips (usually 100-200 µl); and 10^n is the dilution factor.

Packaging of the KUN replicon RNA into transmissible "infectious" particles by the KUN structural proteins expressed from the recombinant SFV replicons. Because the KUN replicon construct C20rep was able to successfully transfect only 10-20% of cells a KUN replicon of greater transfection efficiency was used for attempted packaging in doubly transfected cells (i.e. KUN replicon, and recombinant SFV replicons expressing KUN structural proteins). This significantly improved the efficiency of transfection in BHK21 cells to about 80% using the replicon construct C20DXrep, which was used in all packaging experiments. As noted above, all cell culture fluids from packaging experiments were filtered to remove large membrane fragments and treated with RNase A to remove naked RNA.

Initial cotransfection experiments showed that simultaneous transfection of C20DXrep RNA and SFV RNAs expressing KUN structural proteins did not result

in the detection of infectious particles. Therefore a delay period of 12h or longer between electroporations was used in subsequent experiments to allow KUN replicon RNA to accumulate before electroporation of SFV RNAs. IF and Northern blot analyses of BHK cells infected with the tissue culture fluid collected at 27h after the first electroporation with C20DXrep RNA, and at 26h after the second electroporation with recombinant SFV RNAs, showed higher efficiency of packaging when the single SFV-prME-C RNA was used compared to that obtained with two SFV RNAs, SFV-prME and SFV-C (compare panels 1 and 2 in Fig. 6A, and lanes 1 and 2 in Fig. 6B, respectively). Significantly, IF and Northern blot analysis showed that no released infectious particles containing replication competent SFV RNA were produced when SFV-prME-C RNA alone, or SFV-prME and SFV-C RNAs together, were transfected with (Fig. 6C, lanes 2 and 3) or without previous transfection of KUN replicon RNA. These results clearly demonstrated that the recombinant SFV replicon RNAs containing inserted KUN structural genes could not be packaged by the KUN structural proteins, hence the packaging was specific for KUN RNA. Also, no infectious particles containing packaged KUN replicon RNA were detected when only SFV-prME RNA was used in packaging experiments with C20DXrep RNA (panel 3 in Fig. 6A), demonstrating that coexpression of C protein is absolutely essential for the formation of the secreted infectious particles.

To optimize the conditions for efficient packaging of C20DXrep RNA in cotransfection experiments with SFV-prME-C RNA, variable time points between electroporations (Fig. 7A), and between the second electroporation and harvesting of the infectious particles (Fig. 7B), were examined. Initially optimisation of the time between the two electroporations was studied with a fixed time for collection of the infectious particles. Equal amounts of cells were seeded onto cell culture dishes after the first electroporation with C20DXrep RNA, and cells were subsequently electroporated with SFV-prME-C RNA at 12h, 18h, 24h, or 30h incubation intervals. Culture fluid was then harvested from each dish at 24h after the second electroporation and serial dilutions were used to infect BHK21 cells. IF analysis of these cells with anti-NS3 antibodies indicated a

gradual accumulation of infectious particles from 12h to 24h between electroporations, the highest titer reaching 3.8×10^6 infectious particles at 24h from 2×10^6 of initially transfected cells (Fig. 7A). Northern blot analysis of total RNA from infected cells with a labelled KUN-specific cDNA probe showed that the optimal time interval between the electroporation of KUN replicon RNA and of SFV-prME-C RNA was between 18h and 30h (Fig. 7A). When the interval between electroporations was extended to 36h and 48h the yield of produced infectious particles was reduced.

In a separate study BHK cells were electroporated with SFV-prME-C RNA at 30h after electroporation with C20DXrep RNA and seeded into one 60 mm culture dish. Single aliquot's of the culture fluid (1ml of total 3ml) were then collected at 24h, 30h, and 42h after the second electroporation. The volume of the remaining culture fluid after removal of each aliquot was adjusted to the original volume by adding fresh media, and cells were re-incubated. Collected aliquots were then used to infect BHK cells and total cell RNA recovered from these infected cells at 24h was then analysed for relative amounts of amplified KUN replicon RNA using IF analysis with anti-NS3 antibodies and Northern blot hybridisation with a labelled KUN-specific cDNA probe. The gradual increase in amplified KUN replicon RNA from 24h to 42h after the second electroporation with SFV-prME-C RNA detected by Northern blot analysis (Fig. 7B) was in accord with an increase in released infectious particles as shown by IF analysis of newly infected cells with anti-NS3 antibodies.

Characterisation of the infectious particles. To prove that infectious particles secreted into the culture fluid of cells transfected with C20DXrep and SFV-prME-C RNAs were in fact virus-like particles incorporating KUN structural proteins, a virus neutralisation test was performed. An hour incubation of this tissue culture fluid at 37°C with a 1/10 dilution of the cocktail of monoclonal antibodies to KUN E protein with known neutralising activity resulted in almost complete loss of infectivity (panel 1 in Fig. 8A), while no loss of infectivity was observed in the control sample incubated under similar conditions in the absence of antibodies

(panel 2 in Fig. 8A). Similar results were obtained when incubations with antibodies were performed at room temperature or at 4°C.

To show that the infectious particles can be concentrated by pelleting, a clarified culture fluid of cotransfected cells was subjected to ultracentrifugation. When pellet and supernatant after ultracentrifugation were used to infect BHK cells which were later (at 24h) analysed by IF with anti-NS3 antibodies, virtually all the infectious particles were present in the pelleted material (compare panels 1 and 2 in Fig. 8B).

To identify the proteins and to detect the presence of KUN replicon RNA in the recombinant infectious particles, they were immunoprecipitated in the absence of detergents from the culture fluid of cotransfected and radiolabeled cells using anti-E antibodies. Half of the immunoprecipitated sample was used for separation in the SDS-polyacrylamide gel, and the other half was used to extract RNA by proteinase K digestion. Radioautography of the polyacrylamide gel showed the presence of E, prM, and C proteins in the immunoprecipitates of culture fluid collected from cells either sequentially transfected with C20DXrep and SFV-prME-C RNAs or infected with KUN virus (Fig. 8C, lanes 1 and 3, respectively). E and prM proteins, but no C protein was immunoprecipitated from culture fluid of cells transfected only with SFV-prME-C RNA (Fig. 8C, lane 2), suggesting that specific interaction between KUN replicon RNA and KUN C protein was required for assembly of secreted infectious particles. Note that secreted flaviviruses often contain significant amounts of uncleaved prM as observed in Fig. 8C.

RNA extracted from the immunoprecipitates was reverse transcribed and PCR amplified using KUN-specific primers. A DNA fragment of predicted size (~700 bp, NS2A region) was observed in the RT-PCR reactions of RNAs extracted from the immunoprecipitates of the culture fluid collected from cells either transfected sequentially as in Fig. 6 with both C20DXrep and SFV-prME-C RNAs (Fig. 8D, lane 2) or infected with KUN virus (Fig. 8D, lane 4). No RT-PCR product was obtained from RNA extracted from the immunoprecipitate of the culture fluid

collected from cells transfected with SFV-prME-C RNA alone (Fig. 8D, lane 3). These analyses established that the infectious RNA recovered from packaging experiments was demonstrably packaged in particles encapsidated by the KUN structural proteins.

Example 3

Construction of alternative replicons

KUN replicon vector (C20DX2Arep) was constructed from the vector C20DXrep (details of construction of C20DXrep vector are described above) by insertion into the AscI site of the sequence coding for autoprotease 2A of the foot-and-mouth disease virus (FMDV). Two unique sites for cloning of foreign genes were incorporated: (1.) a SpeI site between the first 20 amino acids of C protein and the 2A sequence, and (2.) a EagI site between the 2A sequence and the rest of the KUN replicon sequence. Cloning into SpeI site ensures the correct cleavage of C20-FG-2A fusion protein from the rest of the KUN polyprotein sequence. Cloning into the EagI site permits correct N-terminus cleavage, but it will have its C-terminus fused to the 22aa of E protein. Transfection with ~5-10 μg of this vector RNA results in ~80% positive cells.

Insertion of heterologous genes such as the GFP gene and the chloramphenicol acetyltransferase (CAT) gene into the SpeI site in this vector resulted in the expression of functionally active proteins.

To separate translation of FG from translation of the downstream KUN polyprotein, a dicistronic C20DXIRESrep vector was constructed by inserting a EMCV IRES sequence into the AscI site. Cloning of the CAT gene upstream of the IRES sequence resulted in the expression of the functionally active CAT protein.

To express FG without fusion to the first 20 amino acids of KUN core protein, the core coding content in the C20DXrep (as well as in the C20DX2Arep and C20DXIRESrep) was eliminated. This was achieved by mutating the native KUN initiation codon thus forcing translation to commence at the second ATG.

The present invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The above-described embodiments are therefore to be considered in all respects as illustrative and not restrictive.

Dated this twenty eighth day of November 1997.

The Crown In The Right Of The Queensland Department Of Health
(Sir Albert Sakzewski Virus Research Centre)
Applicant

Wray & Associates
Perth, Western Australia
Patent Attorneys for the Applicant(s)

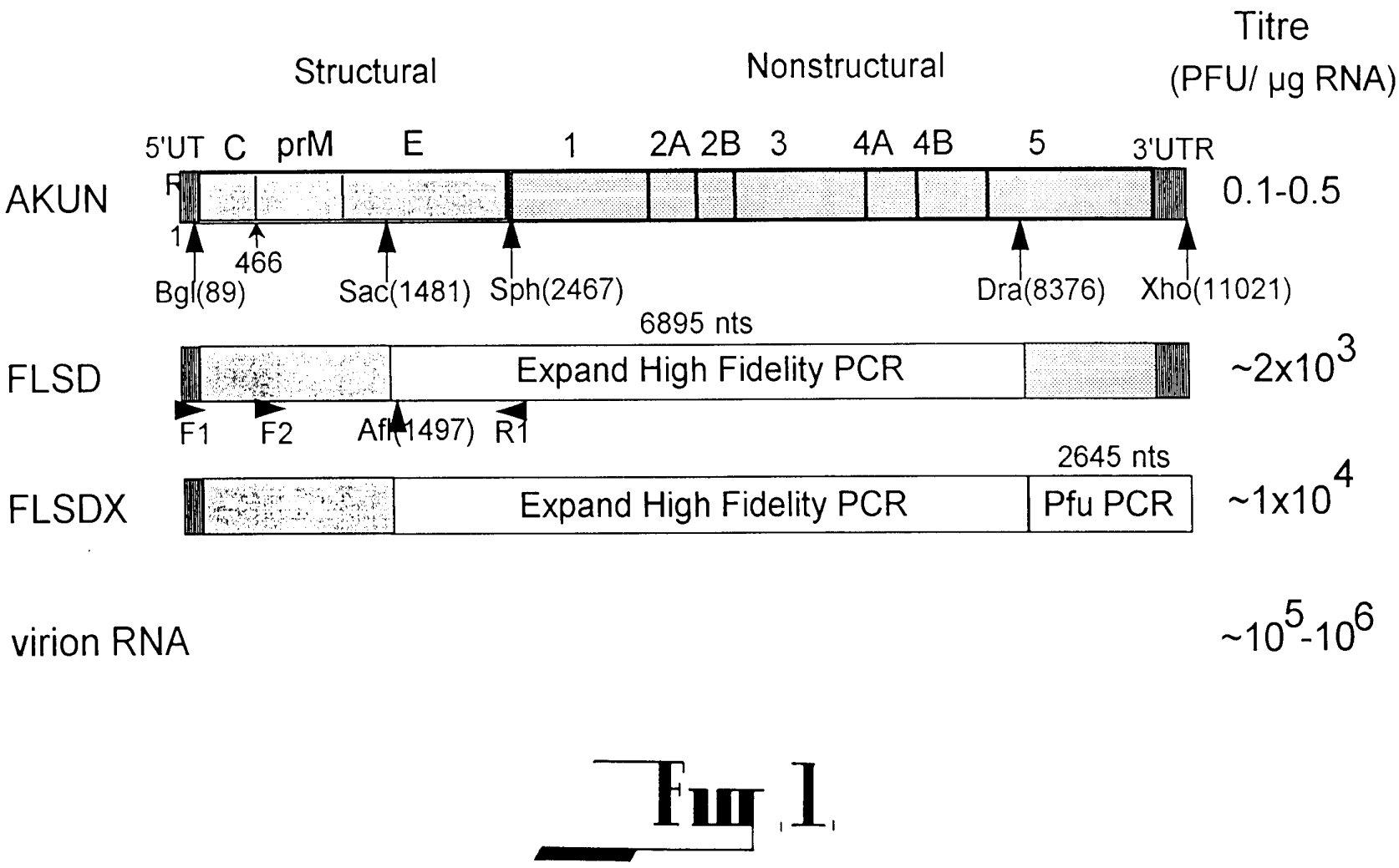
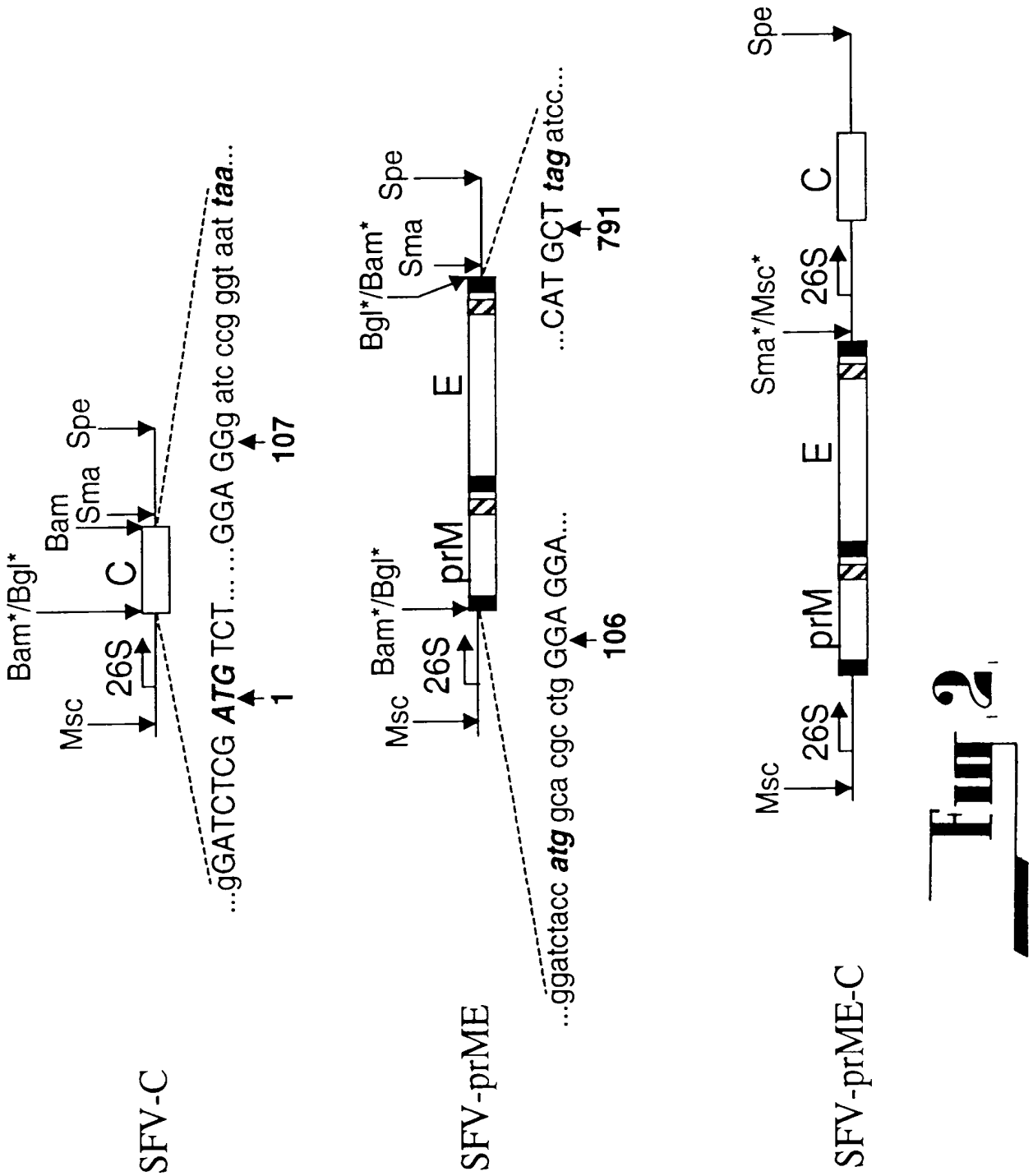

Titre
(PFU/ µg RNA)
Structural
Nonstructural
5'UT C prM E 1 2A 2B 3 4A 4B 5 3'UTR
AKUN
0.1-0.5
1
466
Bgl(89)
Sac(1481)
Sph(2467)
Dra(8376)
Xho(11021)
6895 nts
FLSD
Expand High Fidelity PCR
~2x10^3
F1
F2
Afl(1497)
R1
2645 nts
FLSDX
Expand High Fidelity PCR
Pfu PCR
~1x10^4
virion RNA
~10^5-10^6

Fig 1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Msc
26S
Bam*/Bgl*
C
Bam
Sma
Spe
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1
107
SFV-prME
Msc
26S
Bam*/Bgl*
prM
E
Bgl*/Bam*
Sma
Spe
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Msc
26S
prM
E
Sma*/Msc*
26S
C
Spe

Fig 2

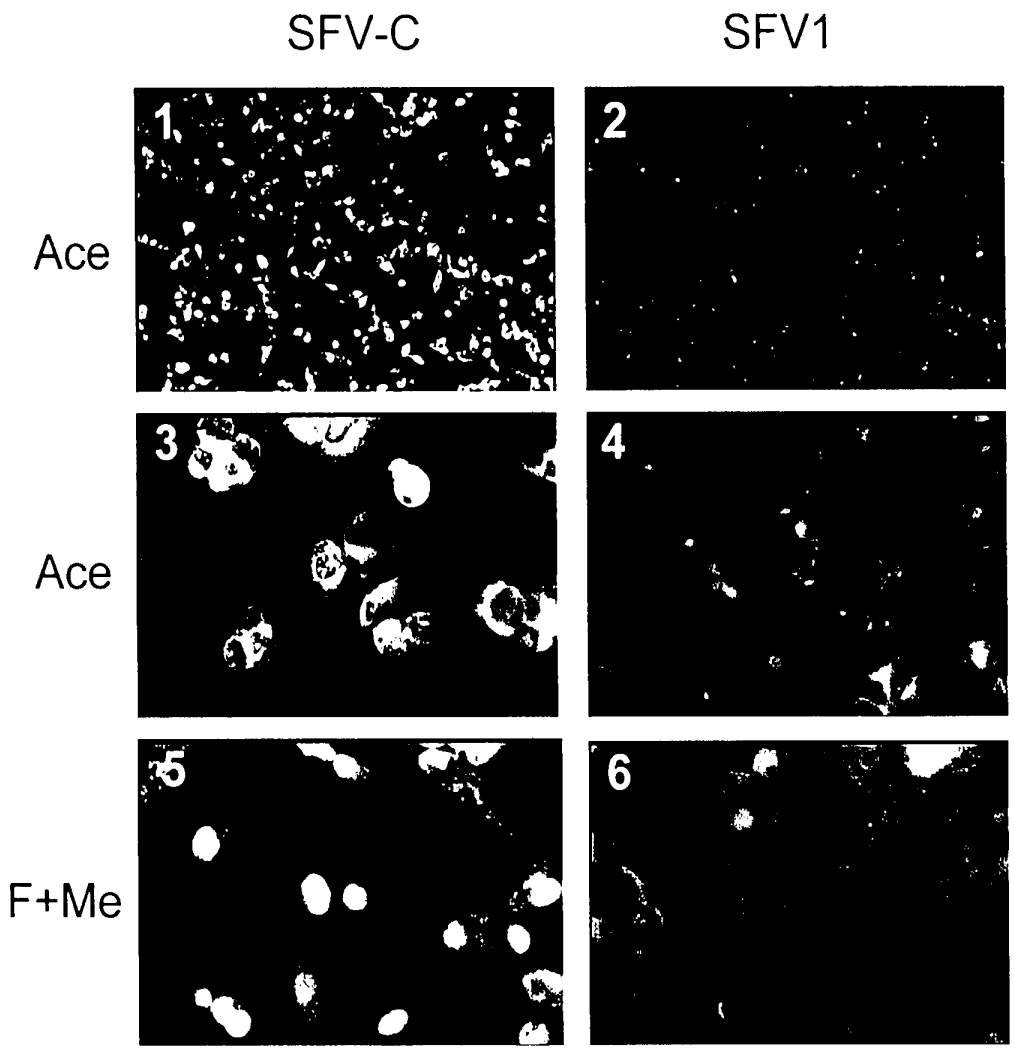
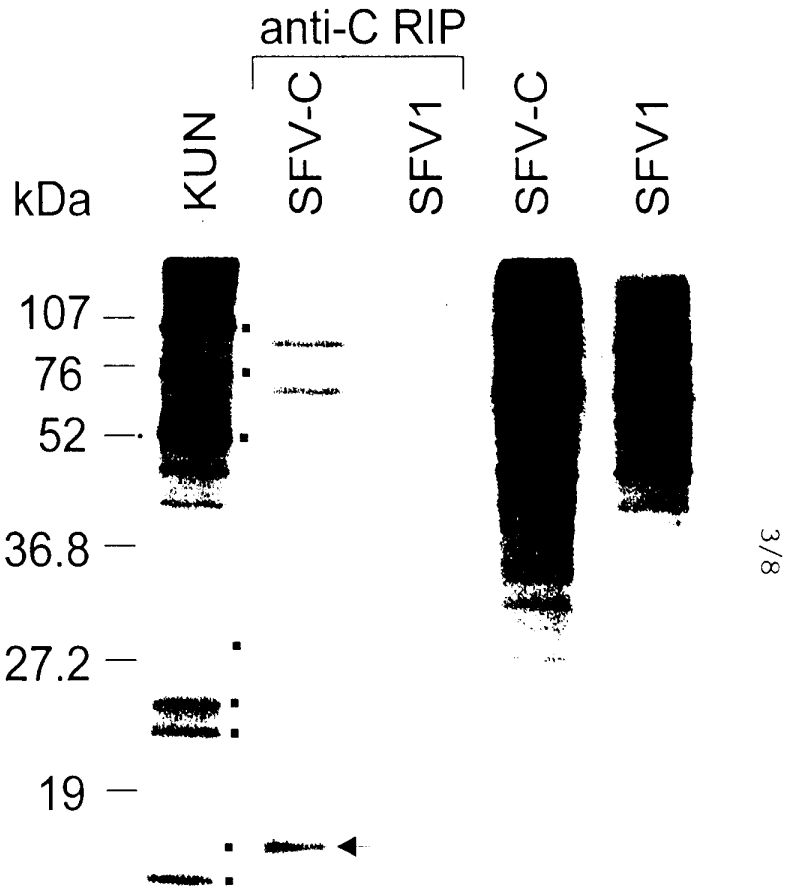

SFV-C
SFV1
Ace
Ace
F+Me
1
2
3
4
5
6

Fig. 3A.


anti-C RIP
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KUN
SFV-C
SFV1
SFV-C
SFV1
107
76
52
36.8
27.2
19

Fig. 3B.

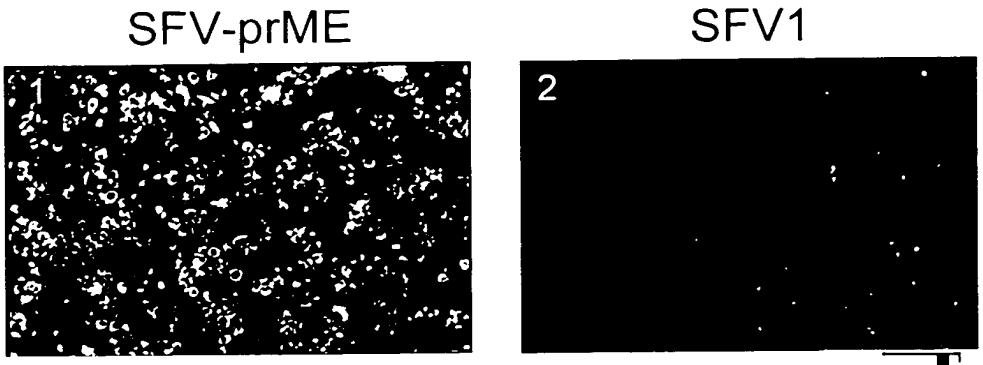


Fig. 4A

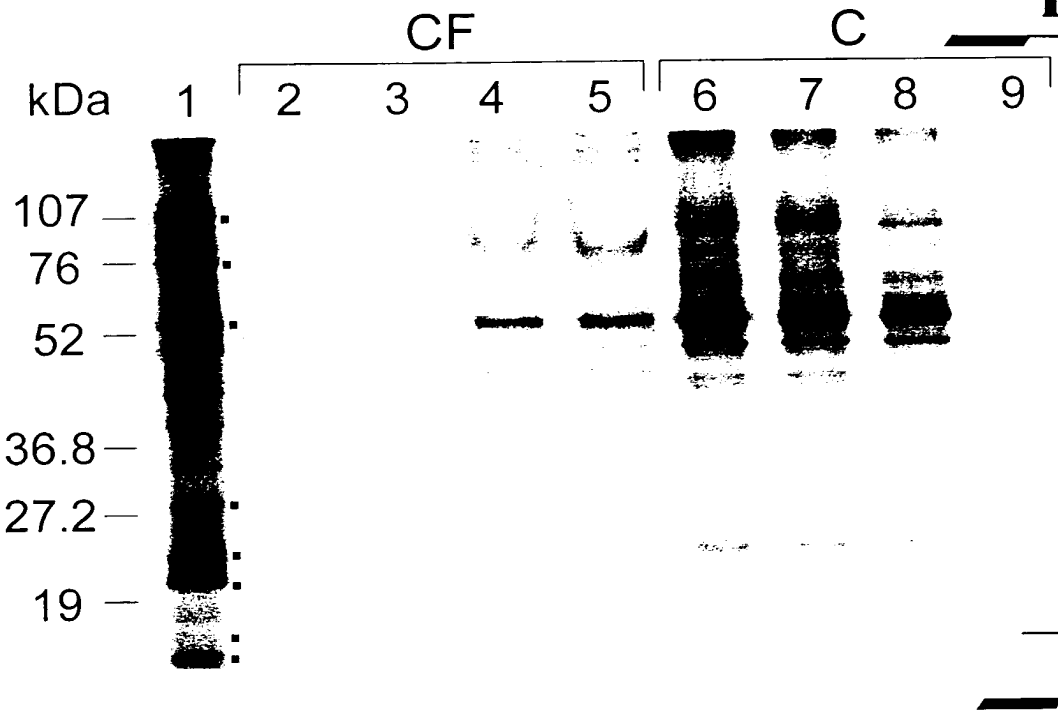


Fig. 4B

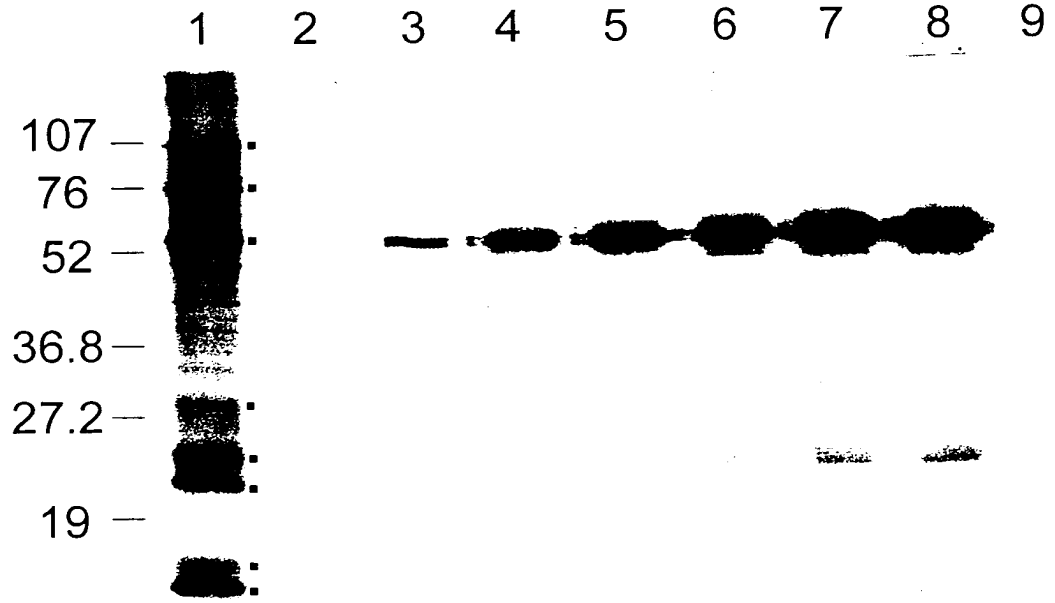


Fig. 4C

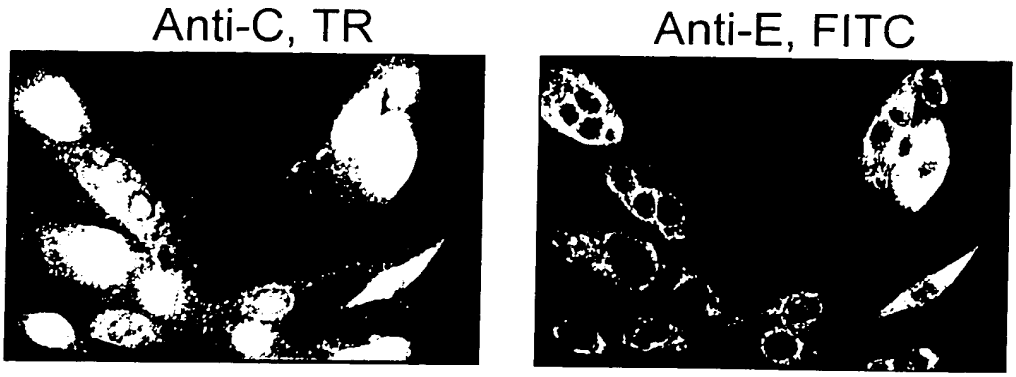


Fig. 5A

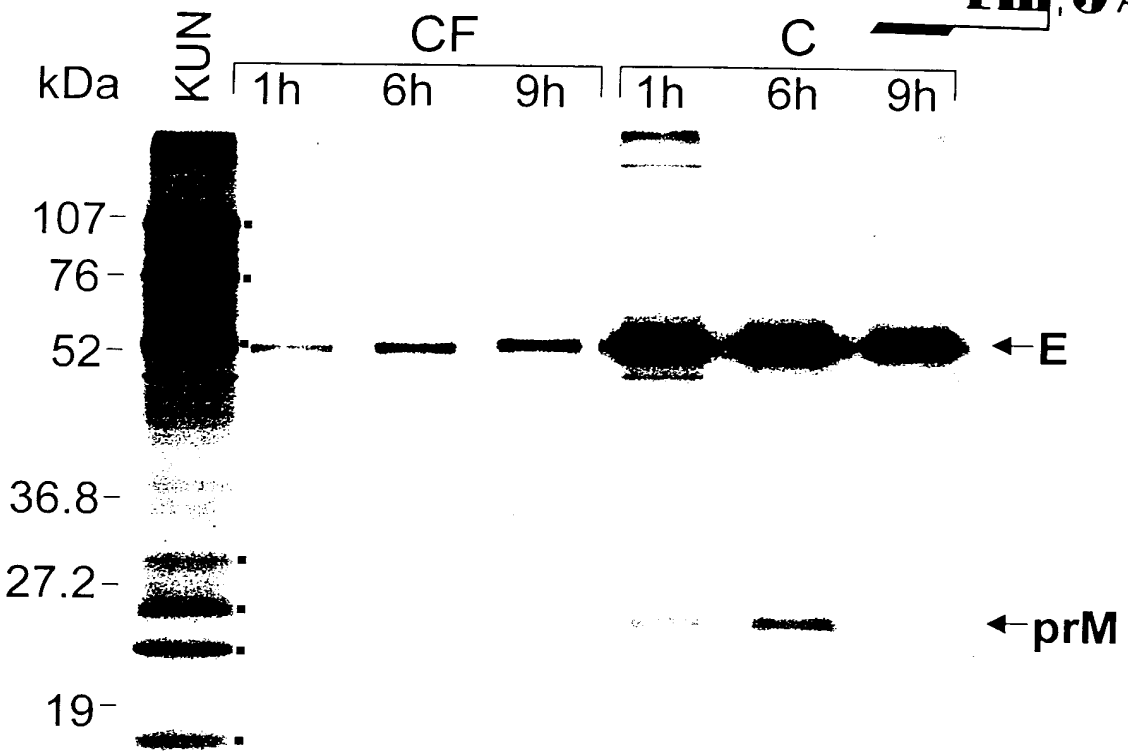


Fig. 5B

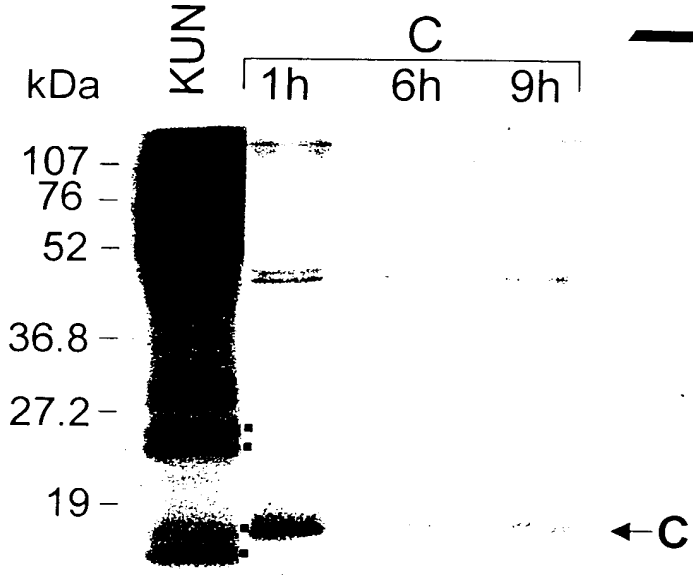
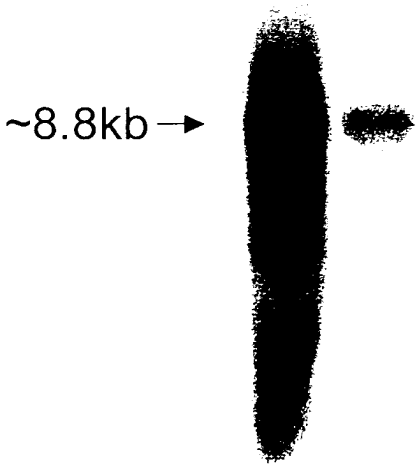


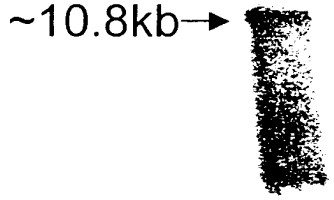
Fig. 5C

1 2 Fig. 6A 1 2 3



KUN probe

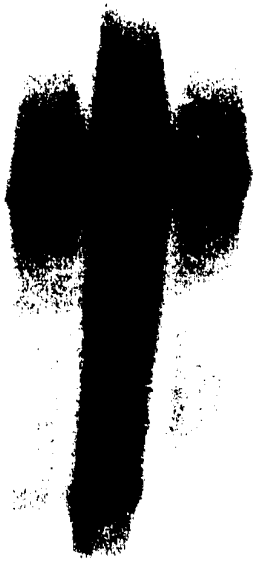
Fig. 6B



SFV probe

Fig. 6C

Time between transfections (h) 12 18 24 30



Titer, $\times 10^6$ (IU/2×10^6 cells) 0.27 2.8 3.8 1.3

Fig. 7A

Time after 2nd transfection (h) 24 30 42

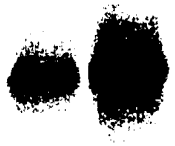


Fig. 7B

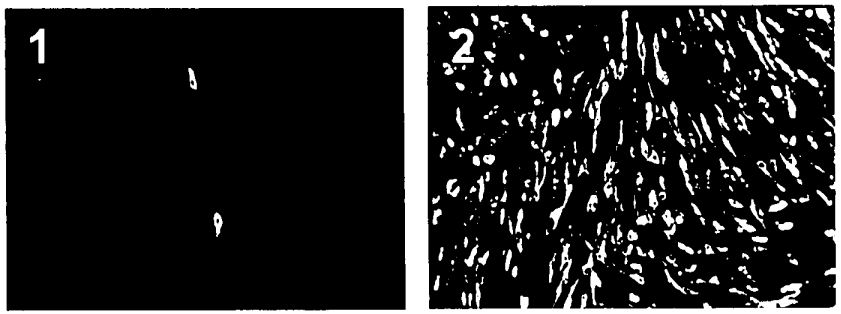


Fig. 8A

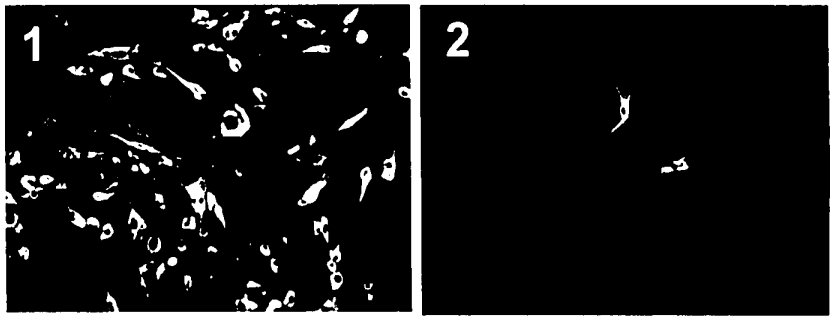


Fig. 8B

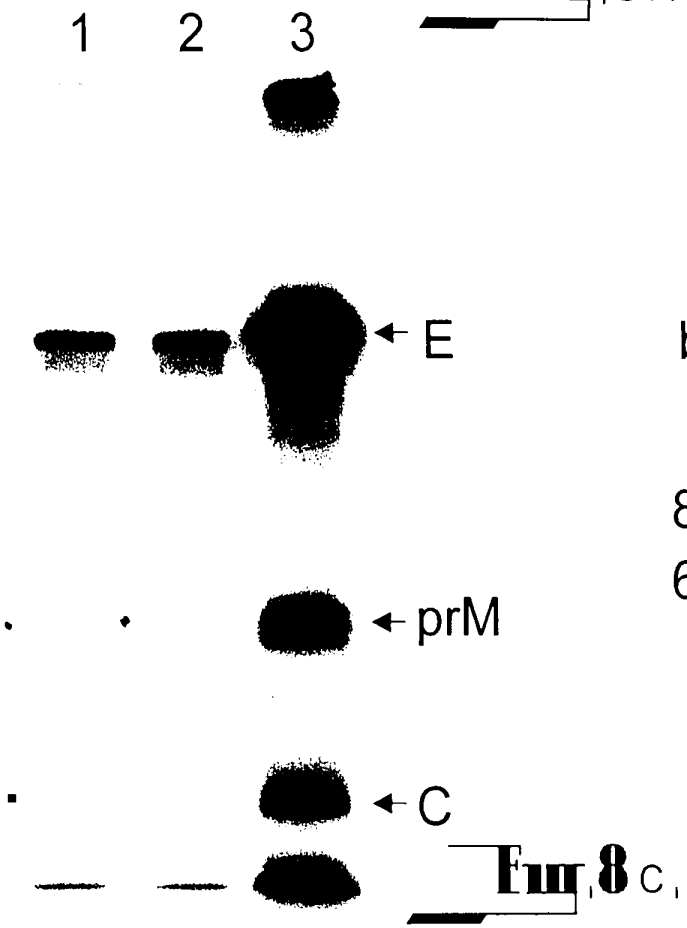


Fig. 8C

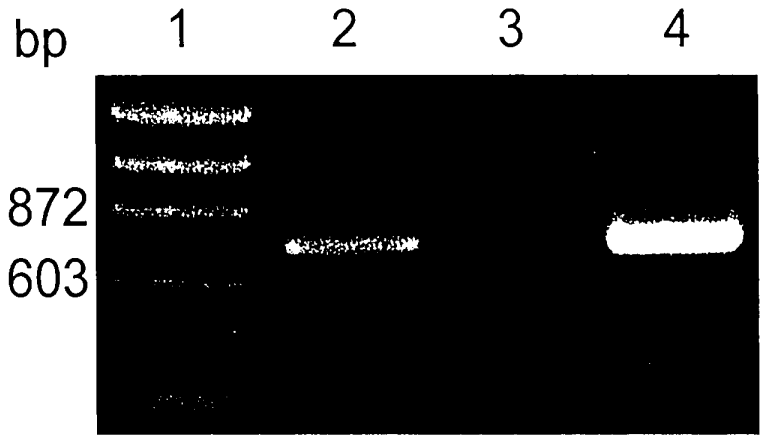


Fig. 8D